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IN RE APPLICATION OF: ROBERT E. JOHNSTON, ET AL. ART UNIT:
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:

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FOR: ALPHAVIRUS RNA REPLICON SYSTEMS

DECLARATION PETER PUSHKO, PH.D., 37 C.F.R. §1.608(b)

ASSISTANT COMMISSIONER FOR PATENTS
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SIR:

I, Peter Pushko, Ph.D., do hereby declare and state:

1. I am a virologist with specialization in developing recombinant viral-based vaccines. I have a Ph.D. in molecular biology from the University of Tartu in Estonia. I worked as scientist at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) where I contributed, through my investigative work, to developing the Alphavirus RNA Replicon Systems in U.S. Patent Application Serial No. 10/683,781 ("the '781 application"). In the process of preparing this declaration I reviewed laboratory notebooks and data summaries including:

Exhibit A Copy of USAMRIID Notebook #3656, VEE Replicon

Exhibit B Data Summary I, pp. 1-4, summary of #3656 experiments, 9/11/94 to 12/26/94

Exhibit C Data Summary II, pp. 1-4, summary of #3656 experiments, 3/22/95 to 7/9/95

2. I understand that some of the notebook page numbers are not visible on the photocopied pages of notebook #3656 (Exhibit A). To aid in the identification of particular pages cited herein, additional numbering was added to each page of the notebook for preparation

of this declaration (e.g., A001, A002, etc., Exhibit A). In addition, my data summaries, which were inserted into my notebook #3656, are reproduced and submitted as separate exhibits (Exhibits B and C).

3. The following is a chronological listing of my laboratory activities related to developing the Alphavirus RNA Replicon Systems disclosed in the '781 application in the time period spanning from prior to November 30, 1994 through May 23, 1995.

4. Prior to and during the aforementioned time period, I conducted research and experimentation as a scientist in the Virology Division of USAMRIID at Fort Detrick in Frederick, Maryland. My activity during this time period was recorded in USAMRIID notebook number #3656 (assigned to Michael Parker, Ph.D., subject identifier "VEE Replicon"; Exhibit A, cover page). The #3656 notebook provides a detailed history of my laboratory activity associated with the development of replication defective infectious alphavirus replicon particles containing heterologous RNA sequences encoding immunogenic proteins and replicon helper constructs required to package the replicon into infectious particles. Entries in notebook #3656 are written by my own hand such that I can attest to their substantive accuracy. In addition, some experiments are documented in typewritten data summaries and I can similarly attest to their accuracy (Exhibit B, Data Summary I; Exhibit C, Data Summary II). A detailed listing of the daily activity involved in each study over this time period is described below.

5. The experimental work reflected by these entries in notebook #3656 occurred predominantly in the V4 portion of the V3V4 Virology Laboratory at Fort Detrick, Building 1425. The V3V4 laboratory suite is a self-contained and safe environment for conducting virology experiments.

6. My experimental work on the Alphavirus RNA Replicon Systems began well prior to November 30, 1994, as is evidenced in entries in notebook #3656 (Exhibit A). This work

included experiments utilizing replicons and replicon helpers that I developed from the genome of an attenuated alphavirus, Venezuelan Equine Encephalitis virus or "VEE." Prior to November 30, 1994, these experiments included developing a VEE replicon particle containing heterologous RNA encoding the nucleoprotein immunogen from Lassa fever virus ("LSN") and the required VEE replicon helper sequences (e.g., Exhibit A, p. A012).

7. Prior to November 30, 1994, methods and assays I employed in developing infectious replication defective replicon particles included: (1) defining transfection protocols for introducing the RNAs into Baby Hamster Kidney (BHK) cells; (2) assessing transfection efficiencies; (3) assaying for expression of Lassa nucleoprotein from VEE replicons and VEE proteins from helper sequences in transfected BHK cells (see, e.g., Exhibit A, p. A024, fluorescent antibody binding to the Lassa N protein); (4) assaying for particle production and infectivity of the particles by incubating media collected from transfected cells with BHK cells; and (5) assaying for expression of the replicon in infected cells by IFA; and (6) assaying for replication competence/incompetence by plaque assay (i.e., harvesting media from infected cells at various time points and incubating the media with permissive cells). (Exhibit A, p. A085).

8. VEE cDNA templates containing attenuating mutations and/or deletions in structural genes (e.g., 272.3/V3059 and pV3014) were utilized for constructing replicon helpers prior to November 30, 1994 (e.g., Exhibit A, pp. A093, A095, A110-111).

9. My notebook entry on November 22, 1994 indicates that I provided samples of my VEE helper construct P5-1 to Dr. Jonathan Smith for the purpose of forwarding the helper construct to our collaborator, Dr. Robert Johnston, at the University of North Carolina at Chapel Hill. (Exhibit A, page A119).

10. The production of infectious replication defective alphavirus particles, as determined by passage on permissive cells, which included a heterologous RNA sequence encoding an

immunogen, had been achieved prior to November 30, 1994 (i.e., October 13-23, 1994 and November 17-25, 1994). Specifically, BHK cells were transfected with replicon helpers, designated P1-5 and P5-1, and a VEE-derived replicon encoding the Lassa fever nucleoprotein immunogen ("LSN"). (Exhibit A, p. A088 and p. A117, respectively). **Infectious particle production for the LSN replicon with the P1-5 or P5-1 helper was calculated (Id., p. A091 and A121, respectively) and LSN antigen expression confirmed by IFA (Exhibit A, p. A094, right hand side, specimen #2).** As determined by passage on permissive cells, no detectable replication competent particles were detected (Id., p. 096, samples 10-13 and p. A122, specimen #33, "0" plaques). These data were summarized in typewritten form and inserted into my notebook (Id., p. A123 and Exhibit B, Data Summary I, p. 3, cell 1, experiment dated October 13, 1994, specimen 2). Transfection efficiency was determined for the second cited example (i.e. P5-1/LSN) using immunofluorescence assay (IFA) with anti-Lassa N and anti-VEE antibodies at two different time points (Exhibit A, p. A118).

11. I discussed these successful results with Jonathan Smith and George Ludwig and provided the infectious replication defective replicon particles encoding LSN to George Ludwig for the first Lassa replicon animal study.

12. DETAILED ACTIVITY RECORD:

November 30, 1994 (Wednesday): The experiments I performed this day were carried forward from the previous day where I had transformed cells with attenuated VEE sequences to create helper constructs P7, P8, P9, and P10. My entry on November 30, 1994 indicates cells transformed with the aforementioned replicon helper constructs were plated. LSN replicons were co-transfected with various replicon helper constructs (i.e., P5-1 and P6-2) at three different concentrations. Helper constructs P5-1 and P6-2 were also transformed in the absence of the LSN replicon as a control. (Exhibit A, p. A126; Exhibit B, Data Summary I, p. 1).

December 1, 1994 (Thursday): I assayed cells transformed on the previous day for expression of LSN antigen and VEE protein using fluorescent-labeled antibodies for LSN antigen and for VEE protein (i.e., IFA using anti-Lassa and anti-VEE antibodies). My entry indicates this experiment was conducted 22 hours after transfection and was used as a measure of transfection efficiency. I also conducted a miniprep isolation of DNA templates of the helper constructs P8 and P10. (Exhibit A, p. A126).

December 2, 1994 (Friday): I performed several IFA's and recorded the production of LSN antigen or VEE protein. Cells transformed with the LSN replicon sequence and P5-1 replicon helper sequence on November 30, 1994 were also assayed for LSN production by IFA and the results documented and transfection efficiencies calculated (Exhibit A, p. A127 and Exhibit B, Data Summary I, p. 1). Miniprep samples were analyzed by gel electrophoresis.

December 3, 1994 (Saturday): No recorded activity in notebook #3656.

December 4, 1994 (Sunday): DNA templates were prepared for in vitro transcription experiments using helper sequences P8-1 and P10-1 and full length VEE sequence (pV3000) linearized with NotI. IFA was also performed for LSN antigen and VEE antigen on samples collected prior to November 30, 1994. (Exhibit A, p. A128).

December 5, 1994 (Monday): I performed plaque assays on twenty different samples using the products from the transfections from November 30, 1994 and November 26, 1994. In vitro transcription for three helper sequences, including P8-1(Δ 1404), P10-1 (Δ 3438), P6-2(Δ 1404) and wild-type pV3000 was also conducted on this day using a slightly modified protocol for transcription of LSN replicon sequence established on November 14, 1994 (Exhibit A, p. A129).

December 6, 1994 (Tuesday): Plasmid DNA preps of p2-20, LSN and 3526 (a double mutant with a deletion in E3 and a substitution in E1) were evaluated by agarose gel electrophoresis and transcription assays were performed on LSN, P5-1 and V3000. (Exhibit A, p. A130).

December 7, 1994 (Wednesday): I recorded the results of the transcription reaction started the previous day and I prepared a restriction enzyme digest of each product (p2-20, LSN and 3526) with ClaI and analyzed the fragments by electrophoresis. Cell transfections were performed using the LSN replicon (prepared 12/6/94) with either the P5-1 or P10-1 helper (prepared 12/6/94 or 12/5/94, respectively). The helpers were also transfected individually (no replicon sequence) and finally, a transfection of the v3014 sequence (triple VEE mutant). A negative control that did not contain any RNA was included. (Exhibit A, pp. A130-A131).

December 8, 1994 (Thursday): I calculated transfection efficiency from the prior day's transfection experiment. The LSN replicon co-transfected with VEE helpers P5-1 or P5-10 was positive for the LSN antigen in 100% of cells, as determined by IFA at 17 hours post-transfection. Also on this day I recorded observations regarding the infectivity analysis of the media from transfected cells by plaque assay of samples using different dilutions. My entries on this day also include gel electrophoresis analysis of VEE 3526 and p2-20 fragments after digestion with certain restriction enzymes. (Exhibit A, p. A131-A133)

December 9, 1994 (Friday): I transformed DH5 α cells with P5-1, P6-2 and LSN (Exhibit A, p. A133).

December 10, 1994 (Saturday): I recorded an entry noting that colonies were picked and grown in three ml of an LB-ampicillin media (Exhibit A, p. A133).

December 11, 1994 (Sunday): I prepared mini-preps using fresh LSTET and STET buffer solutions and dissolved these in 50 ml of sterile water and used 40 microliters in a restriction enzyme digest with NotI at 37°C, taking precautions against contamination. (Exhibit A, p. A133).

December 12, 1994 (Monday): I purified LSN, P5-1 and P6-2 by phenol-chloroform extraction and prepared a transcription reaction for each. (Exhibit A, p. A133).

December 13, 1994 (Tuesday): My entry indicates that I continued plaque assay work from the previous day, although there is no recorded entry referencing this from the previous day. My entry describes extracting RNA from the cell culture medium by the guanidine isothiocyanate method, which is also described in my notebook as "John's Method." RT-PCR was conducted on the RNA samples using concentrations and conditions specified in my notebook. The RNA templates amplified included helpers P1-5, P5-1, and the triple mutant 3014. cDNA primers were either random hexamers or a primer designed to hybridize with the E2 structural VEE gene (supplied by Loreen Hodgson, now Loreen Lofts). The PCR products were separated by gel electrophoresis. (Exhibit A, p. A135).

December 14, 1994 (Wednesday): I transfected cells with the RNA prepared from the transcription reactions of December 12, 1994. Specimen #1 was a co-transfection of the LSN replicon sequence with the P6-2 helper and Specimen #2 was a co-transfection of the LSN replicon sequence with the P5-1 helper. Specimens #3-5 were transfected with LSN, P5-1, or P6-2, respectively. Notations I recorded also reflect that I performed an immunofluorescence assay (IFA) for determining transfection efficiency with antibody to LSN or VEE antigens. Staining of cells transfected with LSN replicon and P5-1 were shown to be expressing the Lassa N antigen (heterologous sequence) and packaging protein from VEE. (Exhibit A, p. A136).

December 15, 1994 (Thursday): I electrophoresed samples of restriction enzyme digested VEE clones 3526 and 3014 (attenuated VEE). My entry notes that these were for use in synthesizing new constructs. Also I prepared transcription reagents for samples of the P8-1 helper, the P10-1 helper, and the LSN replicon. (Exhibit A, p. A137).

December 16, 1994 (Friday): A blot I prepared this day is incorporated into my notebook and partially obscures my notes in the photocopy of this page regarding preparations for reverse transcriptase RT-PCR ("John's method"). A photograph of RNA samples run on an agarose gel

were inserted in the notebook with indication that the RNA samples were from the transfection assay from 12/14/94. (Exhibit A, p. A137).

December 17, 1994 (Saturday): I performed reverse transcriptase (RT) PCR following the same protocol as followed on December 13, 1994. I also performed immunofluorescence assay (IFA) for VEE antigen and recorded that transfections with the LSN replicon and helper sequences were performed, alone or in combination. Infectivity of the supernatant was assayed at 19 hours after infection. Results of an IFA are superimposed over the notes for this day. In addition, new constructs P11 and P12 were created. (Exhibit A, p. A139).

December 18, 1994 (Sunday): I transformed cells with the P11 and P12 helper constructs. IFA analysis was performed and transfection efficiency for LSN-Ag and VEE-Ag was determined. My notes indicate I electrophoresed the products from the RT-PCR and photographed the gel (inserted on page 103 of the notebook, Exhibit A, p. A138). Infectivity analysis was performed and results recorded for the transfection of December 17, 1994. (Exhibit A, p. A140).

December 19, 1994 (Monday): I performed a plaque analysis of the transformations from 12/14/94 and 12/17/94, which had been frozen at -20C. Baby Hamster Kidney (BHK) cells were prepared for the plaque assay. I conducted IFA for infectivity analysis of the transfections from 12/17/94. In addition, I extracted RNA from the transfection of 12/14/94. P12 transformed cells were inoculated into LB media with ampicillin. I also recorded a ligation of the P11 and P12 helper constructs. (Exhibit A, p. A140).

December 20, 1994 (Tuesday): I continued the plaque assay and IFA from the previous day and performed RT-PCR. Plasmid DNAs of the P12 E. coli clones were prepared by miniprep, and a gel of the DNAs cut with enzyme SmaI is shown. A transcription reaction was performed using P10-1 helper and the LSN replicon is documented and I selected mini-prep of P12-1 as helper sequence (3526 cut w/ ApaI and RsrII), with a note that all mini-prep constructs were okay.

(Exhibit A, pp. A140-A141).

December 21, 1994 (Wednesday): I performed transformations with the helper sequences P2-2, P6-3, P8-2, P10-2 (containing various deletions and/or mutations in the E1, E2 and/or E3 genes) and a LSN replicon. I extracted RNA from samples prepared on December 17, 1994 and treated select samples with RNase. Transfection of the helpers specified above, alone or with LSN replicon were performed and transfection efficiency assayed at 16 hours (i.e., LSN and P2-1, LSN and P8-1, LSN and P10-1, LSN replicon, P2-1 helper, P8-1 helper and P10-1). For each of these transfections, the results of infectivity analysis are also recorded indicating they were determined at 15 and 24 hours after infection. Supernatant from transfected cells was collected at 27 hours post-electroporation and plaque analysis outlined for different dilutions. (Exhibit A, pp. A142-A143).

December 22, 1994 (Thursday): I generated and attached a printout from spectrophotometric measurement of a PCR primer. My handwritten concentration calculations are recorded on the printout. The title on the printout is "394 Synthesis Setup Listing" with the date shown at the right as 12/22/94 and the sequence shown from 5' to 3' with the corresponding VEE nucleotide position noted. (Exhibit A, p. A144).

December 23, 1994 (Friday): I conducted RT-PCR for the structural "c" gene on samples from 12/17/94. Infectivity analysis by IFA with LSN and VEE antibodies was performed. My notes indicate I designed PCR primers and questioned designing primers for the nonstructural VEE genes. I prepared minipreps for cleavage with restriction enzyme NotI. Cells were infected using the samples collected on 12/21/94. (Exhibit A, p. A144).

December 24, 1994 (Saturday): I conducted IFA on samples collected 15 and 24 hours post-infection. The NotI cleavage reaction was purified by phenol chloroform extraction and ethanol precipitation and stored at -70C. (Exhibit A, p. A144).

December 25, 1994 (Sunday - Christmas Day): No activity is recorded on this day.

December 26, 1994 (Monday): I performed transcription reactions with different constructs including the LSN replicon and new NotI cleaved P2-2, P8-2, and P12-1 helper sequences. Electrophoresis data is shown. Transfection efficiency was assayed at 20 hours post-electroporation (by IFA with anti-VEE and anti-LSN antibody) for the LSN replicon transfected with either P12-1, P2-2, or P8-2. In addition, each of these four constructs was separately transfected. Media was collected at 28 hours and incubation of the transfected cells continued at 37°C. Also assayed for replicon particle infectivity by exposure of permissive BHK cells with media (supernatant) harvested from transfected cells. Media from the exposed cells was collected at different time points and analyzed by IFA for Lassa N and VEE antigen. (Exhibit A, pp. A144-A146). Plaque assay was performed and noted to be recorded on page 111 (p. A149) of my notebook.

December 27, 1994 (Tuesday) through December 29, 1994 (Thursday): Although no entry appears in the notebook for these dates, it is inherent to the experiments initiated on December 26th that the media from transfected cells was harvested at times approximately 20, 28, and 47 hours after the transfection. BHK cells were infected on December 28th and media was harvested from infected cells at 19 hours post-infection (see, e.g., Exhibit A, pp. A145-A147 and A152). Thus, the cells were maintained throughout this period and media was collected on each day after the transfection/infection and stored for further evaluation as detailed on January 8, 1995.

December 30, 1994 (Friday) through January 7, 1995 (Saturday): No activity is recorded during this period, which includes the New Year's Day holiday. Page 110 of my notebook (Exhibit A, A148), which is sandwiched between entries from December 26, 1994 and January 8, 1995 appears to have a transposed date (i.e., European format) of dated "6/1/95." It was not my

practice to leave a page blank and add notes from dates many months later. Thus, I believe this entry reflects my entry for January 6, 1995, which documents that I performed PCR with LSN replicon and P8-2 helper sequences as template.

January 8, 1995 (Sunday): A plaque assay was performed from the samples generated on December 26, 1994 (LSN, P2-2, P12-1 and P8-2). Supernatant had been collected at different time points after infection and different concentrations (dilutions) of supernatant were evaluated. Transfections were performed using the LSN replicon and P2-2, P6-3 and P8-2 helper constructs and efficiency assayed by IFA for Lassa and VEE antigens. Infectivity was evaluated on samples collected 27 and 50 hours after transfection by IFA. (Exhibit A, pp. A149-150).

January 9, 1995 (Monday) and January 10, 1995 (Tuesday): The entry of January 8, 1995 documents that samples were collected 27 and 50 hours after the transfection experiment. Thus, on January 9-10, 1995, samples for the IFA and plaque assay were collected and the cells maintained. (Exhibit A, pp. A149-150).

January 11, 1995 (Wednesday): No activity is recorded on this day.

January 12, 1995 (Thursday): No activity is recorded on this day.

January 13, 1995 (Friday): The attenuated VEE clone 3014 was cut using two restriction enzymes to create replicon helpers P13, P14, P15, and P16 and the fragments analyzed by gel electrophoresis. (Exhibit A, p. A 151).

January 14, 1995 (Saturday): No activity is recorded on this day.

January 15, 1995 (Sunday): No activity is recorded on this day.

January 16, 1995 (Monday - Martin Luther King Holiday): No activity is recorded on this day.

January 17, 1995 (Tuesday): No activity is recorded on this day.

January 18, 1995 (Wednesday): Additional restriction enzyme cleavage reactions were performed on the attenuated VEE helper templates from January 13, 1995 and analyzed by gel

electrophoresis (P13-1, P13-2, P14-3, P15-7, P15-8, P15-9). (Exhibit A, pp. A151-A152).

January 19, 1995 (Thursday): I performed transcription of the LSN, P13-2, P14-3 and P15-7 constructs. The results of a plaque assay performed using media collected at different time points from transfections of December 21st and 26th, 1994 were recorded at 24 to 65 hours after staining. (Exhibit A, p. A152-A153).

January 20, 1995 (Friday): No activity is recorded on this day.

January 21, 1995 (Saturday): I transfected cells with various VEE constructs, including LSN replicon and P14-3 helper, LSN and P15-7 helper, P13-2 helper alone, P14-3 helper, and P14-7 helper. I also indicate that media samples were taken at three different time points (29, 54 and 72 hours post transfection). A plaque assay was performed on media harvested at different times after transfection with VEE replicons and helpers. Results for specimens 1-20 are listed: 1-12 from January 8, 1995 experiments; 13-20 from December 14, 1994 experiments. **The LSN-expressing replicon reflected in specimens 13 and 20 (LSN replicon created using helper P5-1) were shown to be replication defective by passage on permissive cells in culture.** (Exhibit A, p. A154).

January 22, 1995 (Sunday): No activity is recorded on this day.

January 23, 1995 (Monday): No activity is recorded on this day.

January 24, 1995 (Tuesday): I performed PCR on templates of the 3014 VEE clone, the P13-2 helper, the P14-3 helper, and the P15-7 helper and analyzed the PCR products by gel electrophoresis. I also conducted RNA purification from media transfected with specimen #5 (P14-3) of January 21, 1995. (Exhibit A, p. A155).

January 25, 1995 (Wednesday): I purified RNA from media harvested from cells transfected with different helper constructs. (Exhibit A, pp. A155-A156).

January 26, 1995 (Thursday): No activity is recorded on this day.

January 27, 1995 (Friday): I infected BHK cells with LSN replicon particles (created using the P5-1 helper) at different concentrations. I performed a plaque assay that was to be read and recorded "Sunday," which is identified in my notes as the day following staining of the cells for identifying plaques. (Exhibit A, p. A157).

January 28, 1995 (Saturday): No activity is recorded on this day.

January 29, 1995 (Sunday): The plaque assay set forth on January 27, 1995 was performed and results recorded adjacent to my outline dated January 27, 1995.

January 30, 1995 (Monday): No activity is recorded on this day.

January 31, 1995 (Tuesday): No activity is recorded on this day.

February 1, 1995 (Wednesday): I performed transfections with two different replicons, one containing the heterologous RNA sequence encoding Lassa N and the second containing the heterologous RNA sequence encoding HA antigen (from *Influenza A* virus). The P5-1 and P12-3 helpers were co-transfected with the LSN replicon and P5-1 with HA replicon and each construct transfected alone. (Exhibit A, p. A158).

February 2, 1995 (Thursday) through February 3, 1995 (Friday): There is no entry in my notebook during this period, but the data recorded on the page dated February 1, 1995 (p. A158) indicates that the transfected cells were stained (IFA) 16 hours after transfection to assay transfection efficiency. In addition, medium was collected and cell morphology examined at 21 hours and 33 hours after transfection. Thus, this work was conducted one and two days following the transfection. My observations were recorded on the same page that outlined the transfection of February 1, 1995. (Exhibit A, p. A158).

February 4, 1995 (Saturday) through February 23, 1995 (Thursday): No activity is recorded for these dates.

February 24, 1995 (Friday): My entry on this day identifies an attenuated VEE clone that was

digested with two different restriction enzymes. I performed a gel electrophoresis of various helper constructs derived from the 3014 attenuated VEE clone, including a variant of the P5-1 helper. (Exhibit A, p. A159).

February 25, 1995 (Saturday): No activity is recorded on this day.

February 26, 1995 (Sunday): No activity is recorded on this day.

February 27, 1995 (Monday): No activity is recorded on this day.

February 28, 1995 (Tuesday): No activity is recorded on this day.

March 1, 1995 (Wednesday): I created helper construct P19-1 and also analyzed samples by gel electrophoresis. (Exhibit A, p. A159).

March 2, 1995 (Thursday): No activity is recorded on this day.

March 3, 1995 (Friday): No activity is recorded on this day.

March 4, 1995 (Saturday): I created helper construct P21, which was derived from P5-1. (Exhibit A, p. A159).

March 5, 1995 (Sunday): No activity is recorded on this day.

March 6, 1995 (Monday): I created helper constructs P22 and P23. (Exhibit A, p. A159).

March 7, 1995 (Tuesday): I conducted transcription reactions on the HA and LSN replicon constructs. (Exhibit A, p. A159).

March 8, 1995 (Wednesday) through March 19, 1995 (Sunday): No activity is recorded in my notebook, however my notes in Data Summary II (Exhibit C, "note" at the bottom of 1st cell on p. 1, experiment dated 3/22/95) indicate a transfection was performed on Wednesday, March 15, 1995.

March 20, 1995 (Monday): I prepared transcription reactions of the following constructs: HA replicon, LSN, and helper constructs P5-1, P21-1, P22-1 and P19-1. Aliquots of each reaction

were analyzed by gel electrophoresis after four hours. (Exhibit A, p. A160).

March 21, 1995 (Tuesday): No activity is recorded on this day.

March 22, 1995 (Wednesday) through March 24, 1995 (Friday) : I transfected cells with two different replicons (LSN and HA) and various helper constructs (e.g. 22-1, 21-1, 19-1, and P5-1). Transfection efficiencies were measured using the appropriate antibodies at 20 hours after transfection. Media was collected at 28 and 46 hours post-transfection. (Exhibit C, Data Summary II, p. 1). In addition, on March 24, 1995, I performed transcription reactions on the HA and LSN replicons and helper constructs P5-1, P22-1 and P19-1. (Exhibit A, p. A160).

March 25, 1995 (Saturday) through March 29, 1995 (Wednesday): No activity is recorded on these days.

March 30, 1995 (Thursday) through April 1, 1995 (Saturday): I performed transfections with the HA and LSN replicons with helper constructs P5-1, P22-1 and P19-1. Transfection efficiency was determined by IFA at 20 hours post-transfection and media collected at 27 hours. Cells were exposed to the media from the 27 hour collection and assayed by IFA 19 hours later. Plaque assay was read at 24 hours post infection (4/1/95). (Exhibit C, Data Summary II, p. 1, cell 2 dated 3/30/95).

April 2, 1995 (Sunday): No activity is recorded on this day.

April 3, 1995 (Monday): No activity is recorded on this day.

April 4, 1995 (Tuesday): I created the P23 helper construct. (Exhibit A, p. A161).

April 5, 1995 (Wednesday): No activity is recorded on this day.

April 6, 1995 (Thursday): I performed transcription reactions of the HA replicon, LSN replicon and helper constructs P5-1 and P21-1. (Exhibit A, p. A161).

April 7, 1995 (Friday): No activity is recorded on this day.

April 8, 1995 (Saturday): No activity is recorded on this day.

April 9, 1995 (Sunday): I performed transcription reactions of the LSN and HA replicon and helper P5-1. (Exhibit A, p. A161).

April 10, 1995 (Monday) through April 16, 1995 (Sunday): Two separate particle production experiments were conducted over the course of these days, which are discussed separately below.

1st Experiment: April 10, 1995 through April 15, 1995: I performed transcription of the P23-2 and P21-1 helper constructs. Aliquots of these samples were analyzed by gel electrophoresis. (Exhibit A, A161). Transfections were performed with replicons LSN and HA, alone or together with helper construct P5-1 at different concentrations. IFA was conducted 21 hours post-transfection and media collected at 27 hours for infecting cells. Infected cells were analyzed by plaque assay at 24 and 80 hours post-infection. Thus, the plaque assay required the cells be maintained and involved measurements and observations on April 12 and again on April 15, 1995. **Sample #6 produced LSN particles that were infectious, as demonstrated by IFA for expression of the LSN antigen, and replication defective, shown by passage on permissive cells (plaque assay with <100 pfu/ml at 24 and 80 hours post-infection).** (Exhibit C, Data Summary II, p. 1, cell 3, experiment dated 4/10/85 [sic 95], #6, LSN +P5-1, infectivity and antigen expression col. 8 and plaque assay/replication defective cols. 12-13).

2nd Experiment: April 12, 1995 through April 16, 1995: I transfected cells with the LSN replicon and several helper constructs (P21-1, P23-2, P19-1), either alone or in combination. Transfection efficiency was determined at 21 hours post-transfection (i.e., April 13, 1995) and media collected at 27 hours post-transfection. Cells were infected and IFA performed 16 hours later (April 14, 1995). A plaque assay was performed at 24 (April 14, 1995) and 65 hours (April 16, 1995). (Exhibit C, Data Summary II, page 2, cell 1, dated 4/12/95).

April 17, 1995 (Monday) through April 20, 1995 (Thursday): No activity is recorded during this period.

April 21, 1995 (Friday): I conducted reactions for annealing oligonucleotides CA1 and CA2.

The annealed product was ligated to generate helper construct P24. (Exhibit A, p. A162).

April 22, 1995 (Saturday): No activity is recorded on this day.

April 23, 1995 (Sunday): No activity is recorded on this day.

April 24, 1995 (Monday): No activity is recorded on this day, but inherent in my entry of 4/25/95 is that the product of the ligation reaction from 4/21/95 was transformed into bacteria and plated. Colonies are ordinarily picked 24 hours after plating. (Exhibit A, p. A163).

April 25, 1995 (Tuesday): I picked colonies of P24 transformed cells. I observed and recorded a finding regarding the DNA of sixteen colonies that were analyzed by gel electrophoresis.

Positive P24 samples were purified with a Wizard PCR prep kit. (Exhibit A, p. A163).

April 26, 1995 (Wednesday) through April 28, 1995 (Friday): I performed transfection of BHK cells with the following constructs: LSN and 6-2; LSN and 10-1; LSN and 16-1; LSN and 23-1; LSN alone; and then 6-2 and 10-1; and 16-1 and 23-1 and assayed for efficiency by IFA. (Exhibit A, p. A163). Transfection efficiencies were performed at 22 hours post-transfection and media collected at 27 hours. BHK cells were infected and at 19 hours post-infection analyzed by IFA. Plaque assays were also carried out and infected cells were maintained daily and the plaque assays read at 24 and 80 hours post-infection. (Exhibit C, Data Summary II, p. 2). In addition, on **April 28, 1995** I conducted transcription reactions for the following constructs: LSN replicon, HA replicon, and helpers including P5-1, P24-3 and P21-1. Aliquots of RNA were analyzed by gel electrophoresis. (Exhibit A, A164).

April 29, 1995 (Saturday) through May 3, 1995 (Wednesday): Transfections were performed for the following combinations: LSN and P5-1, LSN and P24-3, LSN and P21-1, LSN and P19-1, LSN with both P24-3 and P21-1, LSN alone, P5-1 and P24-3 and P21-1 and P19-1. IFA was conducted 18 hours post-transfection and media harvested after 25 hours. Permissive cells were

infected and at 21 hours post-infection, IFA performed (April 30 - May 1). Plaque assay was performed on the infected cells and read at 24 hours (May 1) and 65 hours (May 3) post infection.

Specimen #5 (LSN replicon with P24-3 and P21-1) was shown to produce replication defective particles by passage on permissive cells and infected cells expressed the heterologous RNA encoding the immunogen, Lassa fever nucleoprotein. (Exhibit C, Data Summary II, p. 2, cell 3 dated 4/29/95, specimen #5, col. 8, infectivity confirmed by expression of LSN antigen in infected cells and cols. 12-13, replication defective by plaque assay, <100 pfu/ml).

May 4, 1995 (Thursday) through May 7, 1995 (Sunday): No activity is recorded on these days.

May 8, 1995 (Monday): I prepared transcription reactions for constructs N-Rep (Lassa N replicon, LSN); GP-Rep (Lassa glycoprotein replicon); HA-Rep (HA replicon), helper P21-1 and helper P24-3. (Exhibit A, p. A165).

May 9, 1995 (Tuesday): No activity is recorded on this day.

May 10, 1995 (Wednesday): No activity is recorded on this day.

May 11, 1995 (Thursday): No activity is recorded on this day.

May 12, 1995 (Friday): No activity is recorded on this day.

May 13, 1995 (Saturday): I transfected cells with the following replicon and/or helper constructs: N-Rep, GP-Rep and HA-Rep each with two helper constructs P21-1 and P24-3; each replicon alone and a combination of P21-1 and P24-3 (no replicon). The DNA template and aliquots of the RNA used were analyzed by gel electrophoresis. (Exhibit A, p. A166).

May 14, 1995 (Sunday) through May 17, 1995 (Wednesday): At 22 hours after the prior day's transfection, I made observations with respect to cytopathic effect in transfected cells and at 24 hours after transfection I harvested media from the transfected cells. I also assayed transfection efficiency by IFA using the appropriate antibodies. (Exhibit A, p. A166). These data are also

presented in my Data Summary II, Exhibit C, page 3. On May 14, 1995 media was harvested (24 hour time point), plaque assay performed, and read at 24 hours and again 65 hours (May 17) post-infection. **The plaque assay documents that the replicon particles produced by the transfections of May 14, 1995 were replication defective as determined by passage on permissive cells in culture. (e.g., specimen #1 (LSN), specimen #2 (GP, Lassa fever glycoprotein) and #3 (HA, *Influenza A* virus) and IFA confirmed high level antigen expression for each of specimens #1-3. (Exhibit C, p. 3, cell 1, dated 5/14/95, infectivity at col. 8 (Lassa antigens) and 9 (HA antigen) and replication defective col. 12 and 13).**

May 18, 1995 (Monday) through May 23, 1995 (Tuesday): No activity is recorded on these days. It is my recollection that during this time I was preparing a paper for presentation at a regular technology focused meeting (seminar) summarizing the development described in detail above.

May 23, 1995 (Tuesday): I understand that on May 23, 1995, the application maturing into U.S. Patent No. 5,792,462 was filed.

I, Peter Pushko, Ph.D. declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statements may jeopardize the validity of the U.S. Patent Application Serial No. 10/683,781 or any patent issued thereon.

Date

August 20, 2004

Peter Pushko, Ph.D.

Peter Pushko

Pursuant to 37 C.F.R. §1.608(b)

U.S.S.N. 10/683,781

Diligence Timeline

November 27, 1994 through June 3, 1995

LEGEND

USAMRIID:Lab Activity

Creation of VEE Replicon & Replicon Helpers and Infectious Particle Production
Exhibit A, Notebook #3656
Exhibit B, Data Summary I
Exhibit C, Data Summary II
Declaration of Peter Pushko, Ph.D.

***** **Successful Production of Infectious Replication Defective Alphavirus Particles
w/ Heterologous RNA encoding an immunogen**
as determined by passage on permissive cells (plaque assay)

USAMRIID Animal Study with Lassa Antigen Expressing Replicon Particles

Exhibit O, Initial Lassa Replicon Study, Experimental Designator L9412221
Declaration of George V. Ludwig, Ph.D.

Sun 11-27	Mon 11-28	Tue 11-29	Wed 11-30	Thu 12-1	Fri 12-2	Sat 12-3
***** By this date, replication defective alphavirus particles had been produced, as determined by passage on permissive cells. These particles included a heterologous RNA sequence encoding the Lassa fever nucleoprotein (LSN) antigen which was expressed in infected cells. (Exhibit A, pp. A088, A091, A094, A096, A117-118, and A121-122; Exhibit B, p. 3; Pushko Dec. ¶10).	-transcription LSN & p5-1 -ligation of helper constructs	- RNA extraction - helper construct digest and purification	- Lassa replicon and VEE helper transfections -cells transformed with helper constructs plated. ***** Chiron Application 08/348,472 filed in PTO this date (subsequently abandoned & CIP issues as U.S. 6,015,694) *****	-IFA for Lassa antigen & VEE -mini-preps of helper constructs	-DNA digest and isolation; - IFA: LSN and VEE Ag, calculations of transfection efficiency -BHK cells infected with media harvested from transfected cells	

Week of: 12/4/94

Sun 12-4	Mon 12-5	Tue 12-6	Wed 12-7	Thu 12-8	Fri 12-9	Sat 12-10
-replicon helper templates for transcription - IFA for LSN-Ag & VEE ag	-plaque assays, - replicon & helper transcription reactions	-electrophoresis, plasmid DNA, replicon & helper transcription reactions	- transfections, infectivity analysis & plaque assay for replicon and helper constructs - enzyme digests	-calculate transfection efficiencies -transfections: LSN replicon & helper (IFA) -LSN Ag detected - enzyme digests, electrophoresis	-DH5-alpha transformations of p5-1, p6-2, LSN -set up BHK slides, IFA, PCR	-colonies of transformed cells picked, media inoculated, cultures grown

Week of: 12/11/94

Sun 12-11	Mon 12-12	Tue 12-13	Wed 12-14	Thu 12-15	Fri 12-16	Sat 12-17
-mini-preps of transformed cell cultures, enzyme digest of mini-prep products	-purification of templates for LSN replicon & helper constructs P5-1, P6-2 -transcription reactions for LSN, P5-1 & P6-2	-plaque assays, - RNA extraction - RT-PCR for P1-5, P5-1 and triple mutant V3014	-transfect replicon and helper sequences - LSN & VEE Ag measured by IFA; transfection efficiency calculated	-restriction digest, of attenuated VEE clones 3526 & 3014 for prep of new helper constructs -prep of transcription reactions for LSN replicon & helpers P8-1, P10-1	-RNA extractions & electrophoresis	- VEE Ag detection by IFA -infectivity analysis - LSN particles in supernatant -created helper constructs P11 and P12

Week of: 12/18/1994

Sun 12-18	Mon 12-19	Tue 12-20	Wed 12-21	Thu 12-22	Fri 12-23	Sat 12-24
- transformations with helper constructs - LSN and VEE Ag measured by IFA, transfection efficiency calculated - Infectivity analysis of LSN particles	- BHKs prep. for plaque analysis - IFA, particle infectivity analysis - RNA extraction, plasmid - inoculating media with P12 transformed cells - helper ligation-reactions	- plaque assay, IFA continued - RT-PCR, - mini-preps and enzyme digest of helper construct P12, - transcription reactions, LSN & P10-1	- transformations with helper constructs (P2-2, P6-3, P8-2, P10-2) & LNS replicon - RNA extraction and transfection - plaques picked & frozen - IFA of LSN & VEE Ag, infectivity analysis	- spectrophotometry, primer conc. calculations and primer synthesis	- RT-PCR - IFA for LSN & VEE Ag, infectivity analysis - PCR primer design, minipreps & enzyme digests - cells infected with samples collected 12/21/94	- IFA for LSN & VEE Ag, 15 & 24 hr post-infection - enzyme digest & purification
				1st Animal Study Begins: - Mice inoculated with LSN particles (produced from LSN replicon and helper P5-1)	Maintain ongoing Animal Study – Next Step on 12/29/94	Maintain ongoing Animal Study – Next Step on 12/29/94

Week of: 12/25/1994

Sun 12-25	Mon 12-26	Tue 12-27	Wed 12-28	Thu 12-29	Fri 12-30	Sat 12-31
CHRISTMAS DAY	- replicon & helper transcription - IFA LSN & VEE ag, transfection efficiency cacl.	- media harvested at 20 & 28 hours post-transfection - plaque assay, BHK infected w/ 28 hr media	- media harvested at 47 hours post transfection - plaque assay, BHK infected w/47 hr media - media harvested 19 hrs post-infection	- media harvested at 65 hours post-transfection - media harvested at 19 hours post-infection		
Maintain ongoing Animal Study – Next Step on 12/29/94	Maintain ongoing Animal Study – Next Step on 12/29/94	Maintain ongoing Animal Study – Next Step on 12/29/94	Maintain ongoing Animal Study – Next Step on 12/29/94	- 1st Bleed , 7 days post-inoculation	Maintain ongoing Animal Study – Next Step on 1/5/95	Maintain ongoing Animal Study – Next Step on 1/5/95

Week of: 1/1/1995

Sun 1-1	Mon 1-2	Tue 1-3	Wed 1-4	Thu 1-5	Fri 1-6	Sat 1-7
NEW YEAR'S DAY Maintain ongoing Animal Study – Next Step on 1/5/95	Maintain ongoing Animal Study – Next Step on 1/5/95	Maintain ongoing Animal Study – Next Step on 1/5/95	Maintain ongoing Animal Study – Next Step on 1/5/95	2nd Bleed 14 days post-inoculation	Performed PCR: LSN replicon and p8-2 helper templates Maintain ongoing Animal Study – Next Step on 1/12/95	Maintain ongoing Animal Study – Next Step on 1/12/95

Week of: 1/8/1995

Sun 1-8	Mon 1-9	Tue 1-10	Wed 1-11	Thu 1-12	Fri 1-13	Sat 1-14
- cont. analysis of transfection of 12/26/94, IFA for LSN & VEE Ag. Infectivity calculated. - transfections, LSN replicon and helper constructs (P2-2, P6-3, P8-2),	- media harvested at 27 hours post-transfection for IFA & plaque assay	- media harvested at 50 hours post-transfection for IFA & plaque assay			- create replicon helpers (P13-P16), restriction digest of attenuated VEE clone V3014, gel electrophoresis	
Maintain ongoing Animal Study – Next Step on 1/12/95	Maintain ongoing Animal Study – Next Step on 1/12/95	Maintain ongoing Animal Study – Next Step on 1/12/95	Maintain ongoing Animal Study – Next Step on 1/12/95	3rd Bleed 21 days post-inoculation	IFA done with LASSA Spot Slides Assay done by Pushko	Maintain ongoing Animal Study – Next Step on 1/15/95

Week of: 1/15/1995

Sun 1-15	Mon 1-16	Tue 1-17	Wed 1-18	Thu 1-19	Fri 1-20	Sat 1-21
Start ELISA Plates for VEE and Lassa Serology	HOLIDAY – MLK Day		-restriction digest of attenuated VEE helper templates from 1/13/95 -transcription of LSN replicon and 3 helper constructs. Plaque assay on media from 12/21/94	- transcriptions w/ LSN and helper constructs (P14-3, P15-7, P13-2) - plaque assay, from transfection on 12/26/94 and 12/21/94		***** - transfections, LSN & helpers P13-2, P14-3, P15-7 -plaque assays, samples from 12/14/94 & 1/8/95 specimens #13 & #20 (particles produced from LSN/P5-1) were infectious, replication defective, as shown by passage on permissive cells in culture (Ex. A, p. A154) Maintain ongoing Animal Study – Next Step on 1/24/95
	Complete Lassa/VEE ELISA	Maintain ongoing Animal Study – Next Step on 1/18/95	-4th Bleed: 28 days post inoculation 2nd Inoculation (boost at 28 days)	Maintain ongoing Animal Study – Next Step on 1/24/95	Maintain ongoing Animal Study – Next Step on 1/24/95	
	1st positive <i>in vivo</i> immune response to LSN antigen					

Week of: 1/22/1995

Sun 1-22	Mon 1-23	Tue 1-24	Wed 1-25	Thu 1-26	Fri 1-27	Sat 1-28
Maintain ongoing Animal Study – Next Step on 1/24/95	Maintain ongoing Animal Study – Next Step on 1/24/95	- PCR on attenuated VEE clone V3014 and various helper constructs, electrophoresis RNA purification from media of transfected cell specimen #5 of 1/21/95	-RNA purification from media of transfected cell Complete ELISA Lassa/VEE	5th Bleed 35 days post inoculation (PI)/7 days after boost	Maintain ongoing Animal Study – Next Step on 2/2/95	Maintain ongoing Animal Study – Next Step on 2/2/95
					-infect BHK cell w/ LSN particles and outline plaque assay	

Week of: 1/29/1995

Sun 1-29	Mon 1-30	Tue 1-31	Wed 2-1	Thu 2-2	Fri 2-3	Sat 2-4
- plaque assay a performed as set forth 1/27/95			- transfections with HA and LSN replicons and helpers P5-1, P12-3.	- IFA on transfected cells for VEE, HA & LSN Ag, transfection efficiency calculated - media harvested at 16 hrs post-transfection, cell morphology evaluated at 16 hrs	-cell morphology evaluated at 33 hrs post transfection	
Maintain ongoing Animal Study – Next Step on 2/2/95	Maintain ongoing Animal Study – Next Step on 2/2/95	Maintain ongoing Animal Study – Next Step on 2/2/95	Maintain ongoing Animal Study – Next Step on 2/2/95	6th Bleed 42 days PI / 14 days after boost	Maintain ongoing Animal Study – Next Step on 2/9/95	Maintain ongoing Animal Study – Next Step on 2/9/95

Week of: 2/5/1995

Sun 2-5	Mon 2-6	Tue 2-7	Wed 2-8	Thu 2-9	Fri 2-10	Sat 2-11
Maintain ongoing Animal Study – Next Step on 2/9/95	Maintain ongoing Animal Study – Next Step on 2/9/95	Maintain ongoing Animal Study – Next Step on 2/9/95	Maintain ongoing Animal Study – Next Step on 2/9/95	7th Bleed 49 days PI /21 days after boost Start ELISA VEE/Lassa Serology	Complete ELISA VEE/Lassa 2nd measure of positive anti-LSN immune response <i>in vivo</i>	Maintain ongoing Animal Study – Next Step on 2/14/95

Week of: 2/12/1995

Sun 2-12	Mon 2-13	Tue 2-14	Wed 2-15	Thu 2-16	Fri 2-17	Sat 2-18
Maintain ongoing Animal Study – Next Step on 2/14/95	Maintain ongoing Animal Study – Next Step on 2/14/95	Plaque Reduction Neutralization Test of Serum Samples against V3000	Maintain ongoing Animal Study – Next Step on 2/20/95	Maintain ongoing Animal Study – Next Step on 2/20/95	Maintain ongoing Animal Study – Next Step on 2/20/95	Maintain ongoing Animal Study – Next Step on 2/20/95

Week of: 2/19/1995

Sun 2-19	Mon 2-20	Tue 2-21	Wed 2-22	Thu 2-23	Fri 2-24	Sat 2-25
Maintain ongoing Animal Study – Next Step on 2/20/95	-8th Bleed 60 days PI/32 days after boost	Maintain ongoing Animal Study – Next Step on 2/20/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95

Week of: 2/26/1995

Sun 2-26	Mon 2-27	Tue 2-28	Wed 3-1	Thu 3-2	Fri 3-3	Sat 3-4
Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95
			- created helper construct P19-1 - fragment analysis by gel electrophoresis			- modified helper P5-1 to create helper P21

Week of: 3/5/1995

Sun 3-5	Mon 3-6	Tue 3-7	Wed 3-8	Thu 3-9	Fri 3-10	Sat 3-11
	- create helper constructs P22 and P23	- transcription reactions, HA and LSN replicons				
Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95

Week of:3/12/1995

Sun 3-12	Mon 3-13	Tue 3-14	Wed 3-15	Thu 3-16	Fri 3-17	Sat 3-18
Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	- transfection using reduced cap:GTP ratios (Ex. C, p. 1, note at bottom of cell 1) Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95

Week of: 3/19/1995

Sun 3-19	Mon 3-20	Tue 3-21	Wed 3-22	Thu 3-23	Fri 3-24	Sat 3-25
	-transcription reactions: HA replicon, LSN replicon, helper constructs P5-1, P21-1, P22-1, P19-1		- transfection with replicons LSN or HA and various helper constructs.	- IFA for LSN, HA, VEE Ag, calc. transfection efficiencies -media collected 28 hrs post-transfection	-media collected 46 hrs post-transfection -transcription reactions: HA replicon, LSN replicon & helper constructs P5-1, P22-1, P19-1.	
Maintain ongoing Animal Study – Next Step on 3/21/95	Maintain ongoing Animal Study – Next Step on 3/21/95	Testing of Lassa ELISA Ag dilution Maintain ongoing Animal Study –	Testing of Lassa ELISA Ag dilution Maintain ongoing Animal Study –	Maintain ongoing Animal Study – Next Step on 3/26/95	Maintain ongoing Animal Study – Next Step on 3/26/95	Maintain ongoing Animal Study – Next Step on 3/26/95

Sun 3-26	Mon 3-27	Tue 3-28	Wed 3-29	Thu 3-30	Fri 3-31	Sat 4-1
Start Lassa ELISA Maintain ongoing Animal Study –	Complete Lassa ELISA Maintain ongoing Animal Study –	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95
				- transfections with LSN replicon, HA replicon, various helper constructs, including P5-1.	- transfection efficiency by IFA at 20 hrs post- transfection (HA, LSN, VEE Ag) - media harvested at 27 hrs and BHK infected.	- IFA at 19 hrs post-infection, plaque assay at 24 hrs

Sun 4-2	Mon 4-3	Tue 4-4	Wed 4-5	Thu 4-6	Fri 4-7	Sat 4-8
		- create & analyze helper p23		-transcriptions: HA replicon, LSN replicon & helper constructs P5-1, P21-1		
Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95

Week of:4/9/1995

Sun 4-9	Mon 4-10	Tue 4-11	Wed 4-12	Thu 4-13	Fri 4-14	Sat 4-15
- transcription reactions, HA & LSN replicon, helper P5-1	Experiment. #1 - transfection w/ LSN replicon, HA replicon & P5-1 at different ratios of replicon:helper - transcription reaction, helpers P23-2 & P21-1	Experiment #1 - IFA for LSN, HA, & VEE Ag, transfection efficiency calc., 21 hrs post-transfection - media harvested at 21 hrs post-transfection (4/10), infect BHK cells	Experiment #1 -infected cells (4/11) negative for replication at 24 hrs pi by plaque assay -IFA positive for LSN Ag & negative for VEE - -Experiment #2 - transfections w/ LSN & helper constructs P21-1, P23-2, P19-1, alone or in combination.	Experiment #1 -maintain infected cells for plaque assay until 4/15 (60 hrs post-infection) - --Experiment #2 - IFA for LSN, HA, & VEE Ag, transfection efficiency calc., 21 hrs post-transfection - media collected 27 hrs post-transfection, cells infected	Experiment #1 -maintain infected cells for plaque assay until 4/15 (60 hrs post-infection) - -Experiment #2- - IFA for VEE & LSN Ag at 16 hrs post-infection negative, infectivity analysis -plaque assay, 24 hrs post-infection, maintain cells until 4/16	***** Experiment #1: infectious replic.-defective particles expressing LSN Ag confirmed w/ passage on permissive cells and IFA (LSN particles produced w/ LSN replicon & P5-1 helper; Ex. C, p. 1, cell 3 dated 4/10/85 [sic,95] specimen #6, col. 8 under anti-L and cols. 12-13, <100 pfu, plaque assay) - --Experiment #2 - maintain BHK cells infected 4/13 for plaque assay until 4/16
Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95

Week of: 4/16/1995

Sun 4-16	Mon 4-17	Tue 4-18	Wed 4-19	Thu 4-20	Fri 4-21	Sat 4-22
- --Experiment #2 -infected cells analyzed 60 hrs post-infection (4/13/95) by plaque assay and					- annealing reactions for oligonucleotides, ligation of annealed product to helper P24.	
Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95	Maintain ongoing Animal Study – Next Step on 4/24/95

Week of: 4/23/1995

Sun 4-23	Mon 4-24	Tue 4-25	Wed 4-26	Thu 4-27	Fri 4-28	Sat 4-29
Maintain ongoing Animal Study – Next Step on 4/24/95	- annealing reactions for oligonucleotides, ligation of annealed product to helper P24. Plaque Reduction Normalization test serum samples V3000	- cells transformed w/ ligation product and plated Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	-gel electrophoresis, P24 transformed colonies and purification of DNA - transfection of BHK w/ LSN replicon and various helper constructs. Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	-IFA, LSN & VEE Ag, transfection efficiency calc - media harvested at 27 hrs post-transfection, infect BHK cells Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	- IFA for VEE & LSN Ag, 19 hrs post-infection - plaque assay at 24 hrs post-infection -transcription reactions for LSN, HA, P5-1, P24-3, P21-1. Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	-maintain infected cells for plaque assay until 5/1 ----- -transfection reactions including LSN replicon & helper constructs P24-3 +P21-1, P19-1, P5-1. Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95

Week of: 4/30/1995

Sun 4-30	Mon 5-1	Tue 5-2	Wed 5-3	Thu 5-4	Fri 5-5	Sat 5-6
maintain infected cells for plaque assay until 5/1 ----- - IFA for LSN Ag at 18 hrs post-transfection. - media harvested at 25 hrs post-transfection; BHK cells infected	***** - plaque assay at 80 hours post infection (4/27) ----- - IFA for LSN Ag at 21 hrs post-infection - plaque assay negative at 24 hrs post infection	***** ----- -maintain infected cells for plaque assay on 5/3	***** ----- -plaque assay negative at 65 hrs post infection; infectious replic. defective alphavirus particles expressing LSN Ag in infected cells (Ex. C, p. 2, cell 2 dated 4/26/95, specimen #5, LSN/P24-3/P21-1, col. 8 under anti-L and col. 11 under plaque assay)			
Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95

Week of: 5/7/1995

Sun 5-7	Mon 5-8	Tue 5-9	Wed 5-10	Thu 5-11	Fri 5-12	Sat 5-13
	- transcription reactions for replicons: N-Rep (LSN), GP-Rep (Lassa glycoprotein), HA-Rep and helpers P24-3 & P21-1					-transfected cells w/ N-Rep; GP-Rep HA-REP and helpers P21-1 & P24-3 - gel electrophoresis on aliquots of RNA
Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95

Week of: 5/14/1995

Sun 5-14	Mon 5-15	Tue 5-16	Wed 5-17	Thu 5-18	Fri 5-19	Sat 5-20
transfection efficiencies calculated at 22 hrs post transfection (IFA for LSN, GP, HA) - media harvested at 24 hrs post transfection; BHK cells infected	***** - Infectivity/Ag expression at 22 hrs pi: positive for LSN, GP and HA and negative for VEE - Plaque assay at 24 hrs negative (<100 pfu/ml) **infectious replic. defective alphavirus particles expressing 3 different Ag (Lassa N, Lassa G, and HA) in infected cells; created with helpers P24-3 & P21-1 (Ex. C, p. 3, 5/14/95, #1-3 at cols. 8-9, 10,12-13)	- maintain cells for plaque assay until 5/17	- Plaque assay at 65 hrs negative (<100 pfu/ml) for all three types of replicons (LSN, GP and HA)			
Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95

Week of: 5/21/1995

Sun 5-21	Mon 5-22	Tue 5-23	Wed 5-24	Thu 5-25	Fri 5-26	Sat 5-27
		***** Constructive Reduction to Practice in UNC- CH/USAMRIID Application 09/448,630 filed in PTO this date and issued as U.S. 5,792,462 *****				
Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95

Week of: 5/28/1995

Sun 5-28	Mon 5-29	Tue 5-30	Wed 5-31	Thu 6-1	Fri 6-2	Sat 6-3
	MEMORIAL DAY					
Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95	Maintain ongoing Animal Study – Next Step (final bleed) on 7/11/95